Chapter 2

Restoring cell surface expression of $GABA_B$ receptors: a potential strategy to limit neuronal death in cerebral ischemia

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Abstract

Cerebral ischemia is the leading cause for long-term disability and subsequent mortality in adults due to prolonged massive neuronal death. Major mechanism behind ischemia-induced neuronal death is the excessive release of glutamate upon oxygen and glucose deprivation (OGD) occurring during ischemic stroke. Ischemic overexcitation of neurons downregulates $GABA_B$ receptors that normally modulates excitatory neurotransmission by its slow and prolonged neuronal inhibition. Sustained activation of glutamate receptors increases the intracellular Ca^{2+} concentration enhancing the activity of CaMKII leading to the phosphorylation of $GABA_B$ receptors at S867 in the C-terminal domain of the $GABA_{B1}$ subunit. This sorts the constitutively endocytosed $GABA_B$ receptors to lysosomal degradation instead of recycling them back to the cell surface. In this study, we aim at observing the neuroprotective effects of restoring cell surface expression of $GABA_B$ receptors by interfering with the CaMKII-induced downregulation of $GABA_B$ receptors with short interfering peptides that prevent the interaction of CaMKII with $GABA_{B1}$ subunit and consequently preventing the degradation of $GABA_B$ receptors. Screening of short synthetic peptides homologous to the sequence of the $GABA_{B1}$ C-terminal domain identified one peptide (R1-Pep) that prevented interaction of CaMKII with $GABA_{B1}$ upon sustained activation of glutamate receptors. This R1-Pep prevented glutamate-induced downregulation of $GABA_B$ receptors in cultured cortical neurons and preserved cell surface levels of the receptors. Further, this peptide shows improved cell survival on glutamate treated cultured cortical neurons even when applied 24 h post excitotoxic insult. We expect that the preserved cell surface $GABA_B$ receptors with R1-Pep treatment under ischemic conditions would counteract the excessive neuronal excitation and thus limit neuronal death in-vivo.

Introduction

Stroke or cerebral ischemia is the leading cause for adult disability and death worldwide (Feigin et al., 2014). Obstruction or disruption of blood supply to parts of the brain deprives the region affected of blood oxygen and glucose causing derailment of cellular metabolism and eventually neuronal death (Budd and Nicholls, 2002; White et al., 2000). Currently, the only pharmacological approach to minimize neuronal death is the thrombolytic drug tissue plasminogen activator (tPA), which needs to be administered within the first 3-4.5 hours of the ischemic insult to restore blood flow in the affected tissue (Hacke et al., 2008; Lai et al., 2014; Moskowitz et al., 2010; Veerbeek et al., 2014). Because of the narrow time

window for its application only a minor fraction of ischemic stroke patients can be treated with tPA. So far, more than 1000 clinical trials testing potential neuroprotective agents, targeting numerous pathways contributing to neuronal death in stroke, had been undertaken with little positive outcome (Ginsberg, 2007; O'Collins et al., 2006; Yenari and Han, 2012). Therefore, the development of alternative, safe, and effective interventions to limit cell death after an ischemic insult is imperative.

Excitotoxicity by sustained activation of glutamate receptors is the major mechanism behind progressive neuronal loss in the brain regions affected by the ischemic insult (Lai et al., 2014; Lipton, 1999; Salińska et al., 2005). Rapid decline in blood supply and subsequent oxygen and glucose deprivation depletes ATP in the affected region. This leads to impaired ionic homeostasis, depolarization of neurons and enhanced neurotransmitter release (Kostandy, 2012). A massive release of glutamate over-activates glutamate receptors (NMDA, AMPA, kainate as well as metabotropic glutamate receptors) causing a steep increase in the intracellular Ca^{2+} concentration that triggers apoptotic neuronal death (Arundine and Tymianski, 2003; Attwell et al., 2000; Maier et al., 2010; Szydlowska and Tymianski, 2010). Under physiological conditions, excessive glutamate receptor activity is regulated by the G-protein coupled $GABA_B$ receptors (Chalifoux and Carter, 2011a, 2010). However, this mechanism appears to be impaired under ischemic conditions.

$GABA_B$ receptors are obligate heterodimers consisting of $GABA_{B1}$ and $GABA_{B2}$ subunits. They are activated by the major inhibitory neurotransmitter GABA and mediate their effects via the $G_{i/o}$ class of G-proteins (Jones et al., 1998; Shaban et al., 2006). $GABA_B$ receptors are expressed abundantly in pre- and postsynaptic regions of inhibitory and excitatory neurons thereby regulating neuronal activity (Bettler et al., 2004; Kulik et al., 2003; Margeta-Mitrovic et al., 1999). Presynaptic $GABA_B$ receptors inhibit voltage-gated Ca^{2+} channels (VGCCs) (Mintz and Bean, 1993) to limit transmitter release, whereas postsynaptic $GABA_B$ receptors gate the G-protein coupled inwardly rectifying potassium channels (GIRKs), that hyperpolarizes the membrane and shunts excitatory currents via the induction of slow inhibitory postsynaptic currents (sIPSC) (Andrade et al., 1986; Lüscher et al., 1997). $GABA_B$ receptors are implicated in a wide range of functions including regulating synaptic plasticity (Patenaude et al., 2003; Pinard et al., 2010), neuronal development (Gaiarsa et al., 2011; Gaiarsa and Porcher, 2013) and network activity of neurons (Kohl and Paulsen, 2010; Leung and Peloquin, 2006; Zilberter et al., 1999). Hence, dysfunction and differential regulation of their expression have been implicated in diverse neuropathological

conditions (Benke, 2013; Benke et al., 2015; Kumar et al., 2013) including cerebral ischemia (Huang et al., 2017; Jackson-Friedman et al., 1997; Liu et al., 2015).

Because $GABA_B$ receptors control glutamatergic neurotransmission (Chalifoux and Carter, 2011a), it is expected that $GABA_B$ receptors will counteract overexcitation of neurons during an ischemic insult. As this is not the case, it leads to the question whether the receptors themselves are not able to control the massive overexcitation or if their functionality is compromised under this pathological condition. The observation that persistent activation of $GABA_B$ receptors with the agonist baclofen during and after the ischemic insult is neuroprotective in experimental animal models of ischemia (Babcock et al 2002, Han et al., 2008, Jackson-Friedman et al 1997, Kulinskii et al 2000, Lal et al 1995, Xu et al 2008, Zhang et al 2007) suggests that $GABA_B$ receptors *per se* are able to counteract excitotoxicity but that the level of $GABA_B$ receptor-mediated inhibition under ischemic condition is insufficient. There is evidence that $GABA_B$ receptor expression is reduced after transient cerebral ischemia (Vollenweider et al 2006, Kim et al 2011, Huang et al, 2017), suggesting that diminished $GABA_B$ receptor-mediated inhibition may contribute to excitotoxic cell death. If this is indeed the case, preserving or restoring normal $GABA_B$ receptor expression levels during or after the ischemic insult may be a promising approach to counteract excessive glutamatergic neurotransmission and thereby neuronal death. There are two recent publications supporting this hypothesis by showing that upregulation of $GABA_B$ receptor expression is accompanied with a neuroprotective effect in animal models of cerebral ischemia. Injection of ferulic acid, a component of herbs used in traditional Chinese medicine, enhance $GABA_{B1}$ expression and reduce ischemic cell damage in the ischemic tissue (Cheng et al 2010). The second report shows that mild hypothermia exerts a robust neuroprotective effect and is associated with increased $GABA_{B1}$ expression in the ischemic brain (Kim et al. 2011). However, a causal link between up-regulation of $GABA_B$ receptor subunits and neuroprotection had not been established in these studies.

To restore $GABA_B$ mediated inhibition under excitotoxic conditions it is important to understand the mechanism(s) causing the downregulation of $GABA_B$ receptor. Recent *in vitro* studies revealed a potential mechanism, which downregulates $GABA_B$ receptors under ischemic conditions. Sustained activation of glutamate receptors leads to the massive elevation of intracellular Ca^{2+} via NMDA receptors, L-type VGCCs and mGluR1/5 (Arundine and Tymianski, 2003; Sattler et al., 1998; Wang and Qin, 2010). This triggers the CaMKII-dependent phosphorylation of $GABA_{B1}$ at S867 (Guetg et al., 2010; Kantamneni et

al., 2014b), which sorts the receptors towards lysosomal degradation (Kantamneni et al., 2014b; Maier et al., 2010; Terunuma et al., 2010a; Zemoura et al., 2016). Increased glutamate receptor activation also increases phosphorylation of $GABA_{B2}$ at S783 by AMPK followed by its subsequent dephosphorylation via PP2A (Terunuma et al., 2010b). The phosphorylation of $GABA_{B1}$ by CaMKII and the dephosphorylation by PP2A appear to be key events that trigger downregulation of the receptors by lysosomal degradation since pharmacological inhibition of CaMKII or PP2A, respectively, prevented the loss of $GABA_B$ receptors under excitotoxic conditions. Therefore, interfering with the interaction of CaMKII or PP2A with $GABA_B$ receptors under ischemic conditions is expected to prevent the downregulation of $GABA_B$ receptors and might be a strategy to limit neuronal death.

In this study, we tested this hypothesis by screening for a small interfering peptide that inhibits the interaction of CaMKII with $GABA_B$ receptors. We show that a short peptide sequence derived from $GABA_{B1}$ prevents downregulation of $GABA_B$ receptors in cultured cortical neurons under ischemic conditions and provides neuroprotection.

Materials and Methods:

Antibodies and Drugs:

Primary antibodies used in this study include:

Table:1 List of primary antibodies used in this study along with their experimental purpose and origin

Target	Host	Dilution		Origin
		Immunofluorescence	In-situ	
$GABA_{B2}$-N-terminus	Rabbit	1:25		Custom made by GenScript (Benke et al., 2002)
$GABA_{B1b}$-N-terminus	Rabbit	1:100		Custom made by GenScript (Benke et al., 1999; Fritschy et al., 1999)
$GABA_{B2}$-C-terminus	Rabbit	1:500	1:100	Abcam (ab75838)
$GABA_{B2}$-C-terminus	Mouse	1:250	1:50	Abcam (ab181736)
$GABA_{B1a/b}$	Mouse	1:250	1:100	Abcam (ab55051)
CaMKII	Rabbit	1:1000	1:100	Abcam (ab52476)
Akt (pan)	Mouse	1:250		Cell Signaling
Akt (pSer473)	Rabbit	1:1000		Cell Signaling
GIRK (Anti-K_{ir} 3.2)	Rabbit	1:250		Alomone Labs
GSK3β (pan)	Mouse	1:250		Abcam
phospho-GSK3β(S9)	Rabbit	1:1500		Abcam
HA-tag	Mouse	1:250	1:100	Sigma-Aldrich
ubiquitin Lys48	Rabbit		1:50	Millipore
ubiquitin Lys63	Rabbit		1:50	Millipore
AMPAR-GluR1	Mouse	1:1000		NeuroMab
NMDA-GLUN2B	Mouse	1:1000		NeuroMab
mGluR5	Rabbit	1:1000		Upstate
CaV1.2 L-Type Ca^{2+} channel	Rabbit	1:1000		Alomone Labs
CaV2.2 N-Type Ca^{2+} channel	Rabbit	1:1000		Alomone Labs
CaV2.1 P/Q type Ca^{2+} channel	Rabbit	1:1000		Alomone Labs

Primary antibodies were labelled with secondary antibodies conjugated with either Alexa Fluor 488 (1:1000, Invitrogen), Cy-3 (1:400, Jackson ImmunoResearch), Alexa Fluor 647 or Cy-5 (1:300, Jackson ImmunoResearch).

Drugs

The drugs used in this study include glutamate (50 μM, Sigma-Aldrich) and KN93 (10 μM, Sigma-Aldrich / Tocris Bioscience).

Plasmids

The DNAs used for magnetofection of cultured cortical neurons were: $GABA_{B1}$, HA-tagged $GABA_{B1}$ and $GABA_{B2}$ (Kaupmann et al., 1998, 1997). $GABA_{B1}$(RSAR), in which the ER retention motif was mutated to enable trafficking to the plasma membrane without $GABA_{B2}$ (Pagano et al., 2001). Phosphonull HA-tagged $GABA_{B1}$(S867A) and phospho-mimetic HA-tagged $GABA_{B1}$(S867D) (mutations of $GABA_{B1a}$ were custom-made by GenScript). α1, β2 and γ2 subunit of the $GABA_A$ receptor (Benson et al., 1998). α7 nAchR (α7 nicotinic acetylcholine receptor) (a kind gift from Prof. Dr. Shiva Tyagarajan, University of Zurich).

Interfering peptides

In initial experiments a synthetic peptide was used consisting of 22 amino acids of $GABA_{B1}$ with additional 9 arginines in order to render the peptides cell-permeable (RRRRRRRRRXXXXXXXXXXXXXXXXXXXXX, custom-made by GenScript). Another peptide containing the same set of amino acids, but in a random scrambled order was used as a negative control (RRRRRRRRRXXXXXXXXXXXXXXXXXXXXX, custom-made by GenScript). Both peptides were modified with a N-terminal biotin to enable their fluorescent labelling with Dylight-647 conjugated Streptavidin (Jackson ImmunoResearch). In later experiments, the peptides were N-terminal tagged with a peptide sequence derived from the Rabies Virus Glycoprotein (RVG) (YTIWMPENPRPGTPCDIFTNSRGKRASNGGGG (Kumar et al., 2007)) in addition to the 9 arginine residues and with an N-terminal FITC-fluorophore for identifying the cells that had taken up the peptide.

Cell culture

Primary neurons from the cerebral cortex obtained from E18 embryos of time-mated pregnant Wistar rats were used for the *in vitro* experiments. Cortical neurons were cultured on glass coverslips pre-treated with poly-L-lysine solution (50 µg/mL in PBS) overnight at 37 °C. Poly-L-lysine enhances the adhesion of the neurons to the coverslips. After incubation, the poly-L-lysine solution was removed and the coverslips were washed 3 times with sterile water as excess poly-L-lysine was found toxic to neuronal cultures. The cortices from E18 rat embryos were dissected on ice in PBS with 5.5 mM glucose and antibiotic-antimycotic solution (1:100, Invitrogen¸ 10,000 units/mL penicillin, 10,000 µg/mL streptomycin, 25 µg/mL amphotericin B). Isolated cortices were washed once with PBGA (PBS, 10 mM glucose, 1:100 antibiotic-antimycotic solution). The cortices were cut into small pieces and digested in papain solution (0.5 mg/mL papain, 1 mg/mL bovine serum albumin (BSA), 10 µg/mL DNaseI, 10 mM glucose) for exactly 15 minutes at 37 °C. The papain digested cortical slices were washed twice in complete DMEM medium (DMEM containing 10 % foetal calf serum (FCS) and 1:100 antibiotic-antimycotic solution). Then, 3 mL complete DMEM medium was added and the tissue was gently triturated. The supernatant with the triturated neurons was collected in a new vial and trituration was repeated thrice to obtain the cell suspension. The cell suspension was poured through a 70 µm cell-strainer and the cells were counted with a cell counter (TC20TM, Bio-Rad). The neurons were then plated onto the poly-L-lysine pre-treated coverslips. For all the experiments except the cell survival assay co-cultures of neurons containing both neurons and glia was used that were cultured with NU serum containing growth medium. Pure cultures of primary neurons were prepared by plating the neurons containing NB medium.

For cultures of pure neurons, 200,000 cells per well were plated onto coverslips placed in 24-well-plates in 1 mL complete DMEM medium. After 4 hours of incubation, the DMEM medium was exchanged with 1 ml NB medium (neurobasal medium, 1:50 B27 supplement, 1:100 antibiotic-antimycotic solution). 50 % of the NB medium was replaced weekly with fresh NB medium to promote growth and survival of the neurons.

For co-cultures of neurons and glia cells, 60,000 cells per well were plated onto coverslips placed in 12-well-plates in 2 ml complete DMEM medium and incubated overnight at 37 °C/5% CO_2. After incubation, the DMEM medium was exchanged with 2 ml

NU-medium (MEM with 15 % NU serum, 2 % B27 supplement, 15 mM HEPES, 0.45 % glucose, 1 mM sodium pyruvate, 2 mM GlutaMAX) (Grampp et al., 2008; Maier et al., 2010). Neuron/glia cultures were used after 11-14 days in vitro (DIV) and pure neuron cultures were used after 12-17 DIV.

Transfection of neuronal cultures

For transfection of neurons with plasmids the co-cultures of cortical neurons and glia cells at 11-14 DIV were used. Neurons were transfected with plasmid DNA as previously described by Buerli et al., (2007). 1 mL culture medium from each coverslip-containing well was transferred to a fresh plate. A master mix containing 28 µl OPTI-MEM (Gibco), 2 µl Lipofectamin 2000 (Invitrogen) and 0.2 µl Combimag (OZ Biosciences) per coverslip to be transfected was prepared and incubated at room temperature for approximately 5 minutes. 30 µl of the master mix was added to each tube containing 1 µg DNA to be transfected in 30 µl OPTI-MEM. The tubes containing the plasmid DNA in OPTI-MEM and master mix were centrifuged, vortexed and incubated for 20 minutes at room temperature. Subsequently, 60 µl of the transfection reaction was added to the neurons per coverslip in the culture plate. The plate was then placed on the magnetic plate in the incubator for 60 minutes. After the incubation, the coverslips were transferred to a fresh culture plate containing 1 mL of the old conditioned medium (see above). The cells were then incubated 48 hrs at 37°C/5% CO_2 before being used for experiments.

Transfection of peptides with the 9-arginine tag

Though peptides were designed with a poly-arginine sequence for cell penetration, the cellular uptake of the peptides were very poor even in neurons pre-treated with 50 µM pyrenebutyric acid in PBS (Takeuchi et al., 2006). Hence, the peptides (10 µg/ml) were transfected using magnetofection exactly as described above for plasmid DNA, which resulted in stable and consistent peptide uptake in neurons. In later experiments the RVG-tagged interfering peptides, which were readily taken up by the cells, were directly added to culture medium at a final concentration of 10 µg/ml. The cultures treated with the peptides were incubated overnight at 37°C / 5% CO_2 before being used for immunofluorescence experiments.

Immunofluorescence staining

To study the cell surface expression of $GABA_B$ receptors, cultured neurons were washed with Buffer A (2 mM $CaCl_2$, 2 mM $MgCl_2$, 30 mM glucose, 25 mM HEPES, 5 mM KCl and 119 mM NaCl, pH 7.4). Cells were incubated on ice with the appropriate primary antibody diluted in the Buffer A containing 10% Normal Goat Serum (NGS) or Normal Donkey Serum (NDS) for 2 hrs. The neurons were washed thoroughly with ice-cold Buffer A and were incubated with secondary antibody coupled to a fluorophore for 1 hr on ice. The cells were then washed extensively with Buffer A and the fixed with 4% paraformaldehyde containing 4% sucrose for 15 min at room temperature. After being washed with PBS, the coverslips were either mounted on glass slides or processed for staining of cytosolic proteins.

In order to stain intracellular proteins, cultured cortical neurons were washed once with PBS, fixed with 4% paraformaldehyde containing 4% sucrose for 15 min and permeabilized for 10 min with 0.2% Triton X-100 in PBS. The cells were then incubated with primary antibody diluted in PBS containing 10% NGS or NDS overnight at 4°C. After primary antibody incubation, the cells were washed extensively in PBS and incubated with secondary antibody coupled to either Alexa Fluor 488 (1:800, Jackson ImmunoResearch), Cy-3 (1:400, Jackson ImmunoResearch) or Alexa Fluor 647 (1:300, Jackson ImmunoResearch) for one hour at room temperature. Subsequently, the cells were washed thoroughly with PBS and the coverslips were mounted on glass slides with DAKO fluorescence mounting medium, for analysis by confocal laser scanning microscopy.

Analysis of cell death

For the neuronal survival analysis, pure neuronal cultures grown in neurobasal medium were used. To measure cell death, each coverslip was treated with propidium iodide (10 µM) for 25 minutes. After treatment with propidium iodide, the coverslips were washed 3 times for 5 minutes with PBS and mounted in DAPI containing DAKO fluorescence mounting medium upside down on an object slide. The object slides were stored at 4°C protected from light until analysed by microscopy. For quantification of cell death, the number of total neurons in each image (stained with DAPI) as well as the number of dead neurons stained with propidium iodide was counted. The ratio of dead neurons and total neurons was used for the evaluation.

In-situ proximity ligation assay

The *in-situ* proximity ligation assay allows the quantitative analysis of protein-protein interactions and post-translational modifications of proteins in cells by microscopy (Leuchowius et al., 2010; Söderberg et al., 2006). The technique works with the tagging of target proteins with their corresponding primary antibodies raised in different species, which are then labelled with a pair of oligonucleotide-tagged secondary antibodies (PLA probes) specific to the species of the primary antibody. The oligonucleotides hybridize to the PLA probes only when the two target proteins are positioned in closed proximity to one another (<30 nm). This generates a circular DNA strand that serves as template for a rolling-cycle amplification cascade to create a long DNA strand containing numerous hybridization sites for fluorophore-attached oligonucleotides. The hybridization from each pair of target proteins bound to the PLA probes generates a signal in form of a fluorescent spot, which can be detected by fluorescence microscopy.

In situ PLA was performed using the Duolink kits from Olink Biosciences (now distributed by Sigma Aldrich) according to the manufacturer's instructions. Cultured cortical neurons were washed briefly in PBS and then fixed with 4% paraformaldehyde for 20 mins at room temperature. After fixation, the neurons were washed again briefly with PBS and then permeabilized with 0.2% Triton X-100 in PBS for 15 mins. The neurons were then incubated overnight at 4°C with the appropriate pair of primary antibodies (diluted in PBS containing 5% BSA) of the two target proteins raised in different species. Thereafter, the neurons were washed extensively for 3 times with PBS and were then incubated with PLA probes (PLA probe anti-mouse minus and PLA probe anti-rabbit plus, all diluted 1:5 in 5% BSA/PBS) for 60 min at 37°C. The neurons were washed twice for 5 min with PBS and were then incubated in a pre-heated humidity chamber with the ligation buffer and with the enzyme to catalyse ligation of the probes (diluted 1:5 in H_2O) at 37°C for 60 min. After washing the neurons two times with 10 mM Tris, pH 7.4, 0.15 M NaCl, 0.05% Tween 20 for 5 min, they were incubated with the amplification buffer containing the fluorescently labelled oligonucleotides (diluted to 1:5 in H_2O) along with the secondary antibody for the detection of one of the target proteins for 100 min at 37°C. A final washing for the neurons was done twice with wash buffer containing 0.2 M Tris pH 7.4, 0.1 M NaCl and once with 1 mM NaCl and 2 mM Tris pH 7.4 for 1 min in the dark before mounting the coverslips on glass slides coated with DAKO fluorescent mounting medium.

Neurons were immediately imaged for PLA signals using a confocal laser scanning microscope (LSM 700) equipped with a 40x plan-apochromat oil immersion objective, 1.3 NA (Carl Zeiss AG, Switzerland) as described below. Quantitative analysis of the acquired images was done with the ImageJ software (Version 1.47t). For quantification of the proximity ligation assay, the soma of each neuron was encircled and the number of fluorescent spots inside this encircled area was counted using the ImageJ function "Find maxima". The noise tolerance was set for each experiment separately to ensure that all PLA signals were counted and was kept constant throughout different conditions within the experiment. The PLA signals of neurons were normalized to the expression levels of $GABA_{B1}$ and to the total analysed area of the neurons. In the negative control, one of the primary antibodies was omitted and did not produce any PLA signal.

Confocal Laser Scanning Microscopy

Images from the immunocytochemistry experiments were acquired using the Zeiss LSM 700 or LSM710 using a 40x plan-apochromat oil differential interference contrast objective, 1.3 NA (Carl Zeiss AG, Switzerland) at a resolution of 1024 x 1024 pixels with the pinhole set to 1 airy unit. Laser intensities and detector gain (including digital gain) was set to eliminate bleed through and to ensure that the pixel intensities were not saturated and remain within the dynamic range. Each image was acquired as a z-stack of 5-optical sections with 0.3 µm spacing. The images were processed using ImageJ and analysed as detailed in (Maier et al., 2010). For quantification of cell surface staining, the outer and the inner border of the cell surface were carefully marked. The mean fluorescence intensity value of the inner border was subtracted from the mean intensity value of the outer border, so that only the fluorescence intensity from the cell surface remained. For quantification of the total staining, the outer border of the neuron was outlined and the mean fluorescence intensity was measured.

Statistics

The experimental data were analysed for statistical significance using GraphPad Prism (version 5.04). Data for two conditions was analysed using t-tests (assuming unequal variances, Welsh correction). Data for more than two experimental conditions were analysed by one-way or two-way ANOVA with Bonferroni's or Dunnett's multiple comparison test. The tests used and the corresponding *p*-values are mentioned in the figure legends and the differences between conditions were considered statistically significant when $p<0.05$.

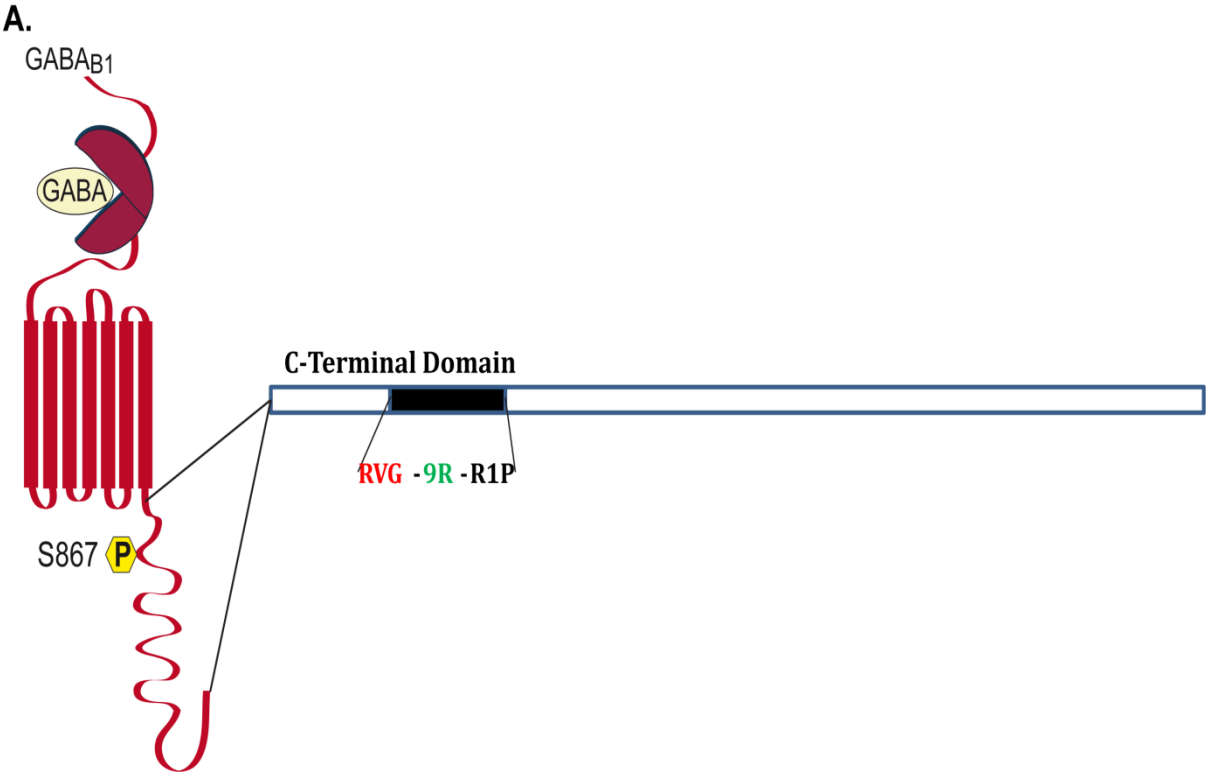
Results

*Identification of a small interfering peptide that prevents the interaction of CaMKII with $GABA_B$ receptors.*For identification of the CaMKII/$GABA_{B1}$ interaction site, peptides of 20-25 amino acids length covering the entire C-terminal domain of $GABA_{B1}$ were synthetized with an additional N-terminal 9 arginine sequence to enhance cell permeability (Fuchs and Raines, 2005). For immunofluorescent detection using Alexa-647-tagged streptavidin all peptides were labelled with biotin at the N-terminus. Unfortunately, the 9-arginine sequence provided inefficient peptide uptake in neurons so that the peptides had to be introduced into the neurons using magnetofection. To screen for interfering peptides, cortical neurons transfected with the peptides were incubated for 60 min with glutamate to induce CaMKII-mediated downregulation of the receptors and tested for improvement in cell surface expression of $GABA_B$ receptors. The screening yielded one peptide comprising the $GABA_{B1}$ sequence (XXXXXXXXXXXXXXXXXXXXX, named R1-Pep) that prevented downregulation of $GABA_B$ receptors (data not shown).

To avoid transfection of peptides into neurons, all subsequent experiments were performed with peptides tagged at their N-terminus with a specific amino acid sequence derived from the Rabies Virus Glycoprotein (RVG) (YTIWMPENPRPGTPCDIFTNSRGKRASNGGGG) (Kumar et al., 2007) (Fig. 1A). This sequence ensures efficient receptor-mediated uptake of the peptides into neurons and also permits penetration of the blood-brain barrier (Kumar et al., 2007). Interestingly, α7 nicotinic acetyl choline receptors (α7-nAChR) (Kumar et al., 2007) and $GABA_B$ receptors (Deshayes et al., 2005) had been suggested to be involved in neuronal uptake of RVG peptides. To test for the mode of peptide uptake, HEK 293 cells were transfected with empty vector, α7-nAChR, α1β2γ2 $GABA_A$ receptor, $GABA_{B1}$(RXRR) ($GABA_{B1}$ with mutated ER retention signal, so that it can traffic to plasma membrane without $GABA_{B2}$), $GABA_{B1a}$, $GABA_{B2}$ and both $GABA_{B1}$ and $GABA_{B2}$ (to form a functional heterodimer). 48 h after transfection, the neurons were incubated for 24 h with RVG-tagged R1-Pep (10 µg/ml) and analysed for receptor expression and the RVG-tagged R1-Pep uptake. Interestingly, RVG-tagged R1-Pep was taken up only by cells expressing $GABA_{B1}$(RXRR), $GABA_{B2}$ or the heterodimeric $GABA_{B1,2}$ receptor, indicating that the peptide entry requires at least one $GABA_B$ receptor subunit at the cell surface (Fig. 1B). The peptide was not taken up by neurons expressing α7-

nAchR, $GABA_A$ or wild type $GABA_{B1}$, that cannot be trafficked to the plasma membrane in the absence of $GABA_{B2}$ (Fig. 1B)

After identification of the mode of cellular uptake of R1-Pep, we tested its efficacy in affecting the cell surface and total $GABA_B$ receptor expression under ischemic-like conditions. As a pilot experiment, we stressed neurons for 60 min with 50 µM glutamate after which the R1-Pep was added to the culture medium at a concentration of 10 µg/ml. The neurons were incubated overnight with the peptide and then examined for cell surface and total expression of $GABA_B$ receptors. Under this condition, R1-Pep increased both cell surface (213±51% of control; Fig 1C) and total (191±54% of control; Fig 1C) expression of $GABA_B$ receptors.



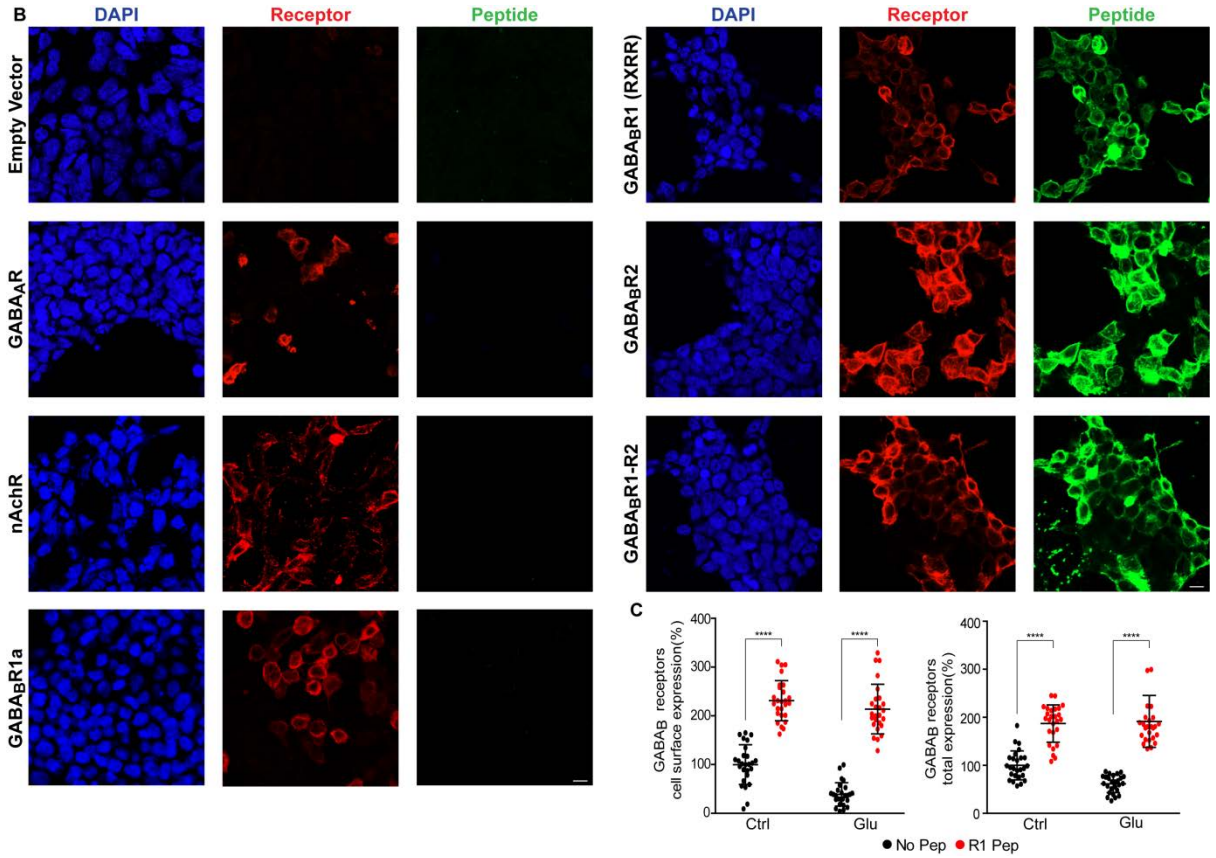


Fig.1. *The RVG-tagged $GABA_{B1}$ sequence (R1-Pep) required cell surface $GABA_B$ receptors for efficient cellular uptake and interfered with glutamate-induced $GABA_B$ receptor downregulation.* A. Sequence and schematic representation of the position of the identified interfering peptide. The interfering peptide was tagged with a peptide sequence derived from the Rabies Virus Glycoprotein (RVG) and in addition with 9 arginine to enable cellular uptake (R1-Pep). For a negative control, a second peptide was synthetized in which the sequence of the R1-Peptide was randomized (Rand-Pep). B. *Mechanism of cellular R1-Pep uptake*. To identify the mode of entry of R1-Pep, HEK cells were transfected with empty vector, α7-nAChR, α1β2γ2 $GABA_A$ receptor, $GABA_{B1}$(RXRR) ($GABA_{B1}$ subunit with ER retention signal mutated, so that it can traffic to plasma membrane without $GABA_{B2}$), $GABA_{B1a}$, $GABA_{B2}$ and both $GABA_{B1}$ as well as $GABA_{B2}$ (to form a functional heterodimer). RVG tagged R1-Pep coupled to FITC was added at a final concentration of 10 µg/ml and incubated overnight at 37°C. The data shows that only those cells expressing cell surface $GABA_B$ receptors efficiently take up R1-Pep. Scale bar 10 µm. C. *R1-Pep prevents glutamate-induced downregulation of $GABA_B$ receptors*. To check the efficiency of R1-Pep in improving cell surface expression of $GABA_B$ receptors, the peptides were introduced into cultured cortical neurons immediately after glutamate stress (60 min with 50 µM glutamate). Cultures not treated with glutamate served as control. Increase in cell surface and total expression of $GABA_B$ receptors was observed in R1-Pep treated neurons. The data represent the mean ± SD of 26 neurons from two different experiments. Two-way ANOVA with Bonferroni's Multiple Comparison test reveleaed interaction between peptide treatments and glutamate stress (cell surface - $F(1,100) = 7.688$, $p=0.0066$; Total – $F(1,100) = 8.50$, $p=0.0044$). The analysis also showed a significant difference in $GABA_B$ receptor expression due to glutamate stress (cell surface - $F(1,100) = 24.36$, $p<0.0001$ and total – $F(1,100) = 5.438$, $p=0.0228$) and with peptide treatment (cell surface - $F(1,100) = 372.8$, $p<0.0001$ and total – $F(1,100) = 216.0$, $p<0.0001$). ****, $p<0.0001$.

R1-Pep prevented the CaMKII-GABA$_B$ receptor interaction and enhanced cell surface expression of GABA$_B$ receptors under ischemic conditions. Upon sustained activation of glutamate receptors, CaMKII interacts with $GABA_B$ receptors and phosphorylates $GABA_{B1}$, which eventually leads to the degradation of $GABA_B$ receptors (Guetg et al., 2010). Since R1-Pep prevented this downregulation in our pilot experiment (Fig. 1), it most likely interferes with the interaction between $GABA_{B1}$ and CaMKII. To test this hypothesis, we analysed the interaction of CaMKII with $GABA_B$ receptors in the presence of R1-Pep using the *in-situ* Proximity Ligation Assay (*in situ* PLA). Cultured cortical neurons were stressed with 50 µM glutamate for 60 min and were then treated with R1-Pep or Rand-Pep (as negative control, randomized R1-Pep sequence). After overnight incubation, the neurons were tested for CaMKII-$GABA_B$ receptor interaction by *in situ* PLA with antibodies raised against CaMKII and $GABA_{B1}$. Treatment of cultured cortical neurons with glutamate increased the interaction between $GABA_{B1}$ and CaMKII (165±73% of control; Fig. 2A). However, incubation of the neurons with R1-Pep considerably reduced the $GABA_{B1}$ and CaMKII interaction in neurons under normal physiological conditions (28±23% of control; Fig. 2A) as well as in neurons stressed with glutamate (20±17% of control; Fig. 2A). The inactive control peptide Rand-Pep neither affected the PLA signal in untreated control neurons (122±51% of control; Fig 2A) nor in glutamate-treated neurons (164±63% of control; Fig. 2A). These results demonstrate the efficiency of R1-Pep to block the interaction between $GABA_{B1}$ and CaMKII.

Because R1-Pep blocks the interaction between $GABA_{B1}$ and CaMKII, we next tested whether this intervention restored or increased cell surface expression of $GABA_B$ receptors. Treatment of cultured cortical neurons with glutamate reduced the cell surface expression of $GABA_B$ receptors (24±18% of control; Fig. 2B). However, the presence of R1-Pep strongly enhanced cell surface expression of the receptors in both unstressed neurons (347±118% of control; Fig. 2B) and glutamate treated neurons (278±95% of control; Fig. 2B), indicating that R1-Pep inhibits degradation of $GABA_B$ receptors under normal physiological and excitotoxic conditions. Incubation with the control peptide (Rand-Pep) neither affected basal cell surface $GABA_B$ expression (100±25% of control; Fig 2B) nor prevented the downregulation under glutamate stress (27±18% of control; Fig. 2B). These findings show that R1-Pep not only blocks the interaction between $GABA_{B1}$ and CaMKII but also increases the cell surface expression of $GABA_B$ receptors.

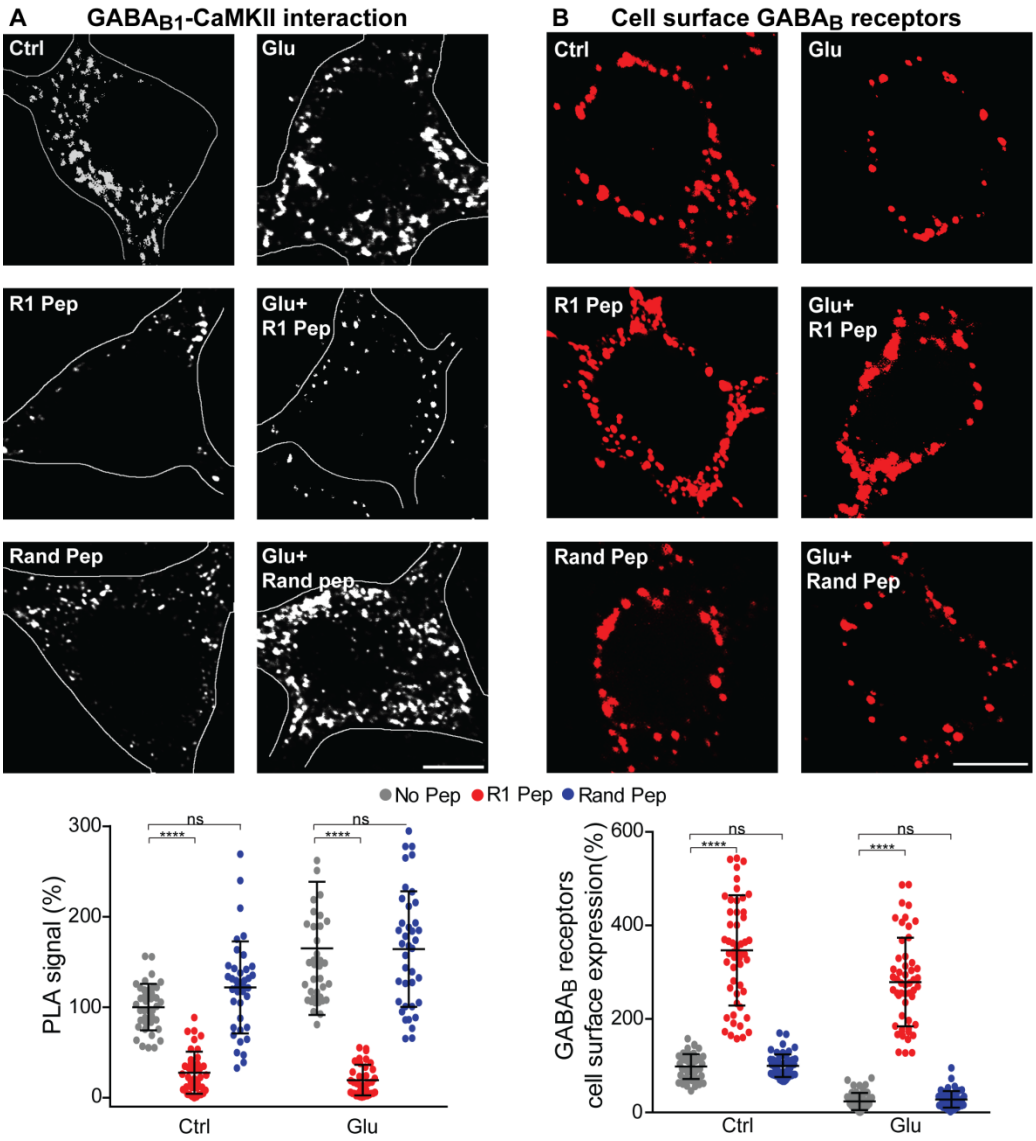


Fig. 2 *R1-Pep blocked the interaction between CaMKII and $GABA_{B1}$ and increased cell surface $GABA_B$ receptor expression* **A**. R1-Pep prevented interaction of CAMKII with $GABA_B$ receptors. Cortical neurons were treated with glutamate (50 µM for 60 mins) and were then immediately treated with R1-Pep or Rand-Pep. The neurons were then incubated for 16 h with the peptides and analysed for the level of interaction of CaMKII with $GABA_B$ receptors by *in situ* PLA using antibodies directed against $GABA_{B1}$ and CaMKII. Top, representative images (*scale bar,* 5 µm). Glutamate treatment increased the interaction between $GABA_{B1}$ and CaMKII (white dots), while incubation with R1-Pep significantly reduced the interaction. Addition of Rand-Pep was without effect. *Bottom*, quantification of *in situ* PLA signals. The data represent the mean ± SD of 39 neurons per condition from three independent experiments. Two-way ANOVA with Bonferroni's Multiple Comparison test reveleaed interaction between peptide treatments and glutamate stress with $F(2,228) = 12.08$, $p<0.0001$. The analysis also showed a significant difference in $GABA_{B1}$-CaMKII interaction due to glutamate stress ($F(1,228) = 28.29$, $p<0.0001$) and with peptide treatment ($F(2,228) = 150.79$, $p<0.0001$). ****, $p<0.0001$. **B**. Interfering with the CaMKII-$GABA_B$ receptor interaction increased cell surface expression $GABA_B$ receptors. The experimental conditions were the same as in A, except that cell surface expression of $GABA_B$ receptors was analysed using $GABA_{B2}$N antibodies. Top, representative images (scale bar, 5 µm). Bottom, quantification of fluorescence intensities. The data represent the mean ± SD of 52 neurons per condition from three independent experiments. Two-way ANOVA with Bonferroni's Multiple Comparison test exhibited no interaction between peptide treatments and glutamate stress with $F(2,306) = 0.08$, $p=0.9270$. However, the analysis showed a significant difference in cell surface $GABA_B$ receptor expression due to glutamate stress ($F(1,306) = 96.30$, $p<0.0001$) and with peptide treatment ($F(2,306) = 524.70$, $p<0.0001$). ****, $p<0.0001$.

R1-Pep dose-dependently reversed CaMKII-induced downregulation of cell surface $GABA_B$ receptors. To determine the potency of R1-Pep in preventing degradation of $GABA_B$ receptors, neurons were treated with 50 µM glutamate for 60 min followed by incubation with increasing concentrations of R1-Pep (0.5, 1.0, 2.5, 5.0, 10, 25 and 50 µg/ml). After overnight incubation with the peptide, the cell surface and total expression of $GABA_B$ receptors was determined. In the absence of peptide, we observed a significant decline in both cell surface (19±10% of control, Fig. 3A) and total (56±22% of control, Fig. 3B) expression of $GABA_B$ receptors. Low doses of R1-Pep (0.5 and 1.0 µg/ml) reversed the effects of glutamate-induced downregulation of cell surface as well as total receptors to control levels (cell surface: 0.5 µg/ml: 74±45%, 1.0 µg/ml: 123±73% of control, Fig. 3A; total: 0.5 µg/ml: 94±25%, 1.0 µg/ml: 117±24% of control; Fig. 3B). With doses of peptides starting from 2.5 µg/ml and higher, we observed a significant increase in cell surface receptor expression (2.5 µg/ml: 173±95%; 5.0 µg/ml: 185±107%; 10 µg/ml: 233±160%; 25 µg/ml: 267±172%; 50 µg/ml: 342±149% of control; Fig. 3A) and in total receptor expression (2.5 µg/ml: 125±31%; 5.0 µg/ml: 136±26%; 10 µg/ml: 150±23%; 25 µg/ml: 154±31%; 50 µg/ml: 171±55% of control; Fig. 3B). These findings indicate a high efficacy of R1-Pep to prevent glutamate-induced downregulation of $GABA_B$ receptors. Since doses of 10 µg/ml provided stable and consistent increase in cell surface expression of $GABA_B$ receptors, this concentration was used in all further experiments.

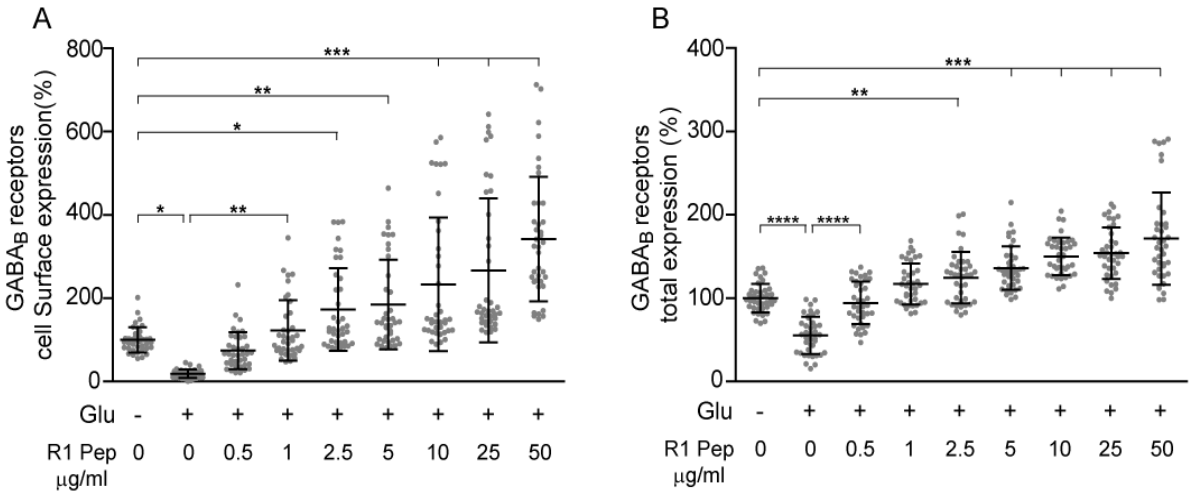


Fig.3 *R1-Pep dose-dependently increased expression of $GABA_B$ receptors after glutamate treatment.* Neurons were stressed with 50 µM glutamate for 60 min followed by incubation of increasing concentrations with R1-Pep (0.5, 1.0, 2.5, 5, 10, 25 and 50 µg/ml). R1-Pep was incubated overnight (16 h) and the neurons were stained for cell surface (A) and total expression (B) of $GABA_B$ receptors. A dose as low as 0.5 µg/ml R1-Pep prevented glutamate-induced downregulation of $GABA_B$ receptors to levels of the untreated controls. However, a significant increase in both cell surface and total expression of $GABA_B$ receptors was observed at 2.5µg/ml and higher. The data represent the mean ± SD of 38 neurons per condition from three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$; one-way ANOVA, Bonferroni's Multiple Comparison test.

R1-Pep treatment did not affect CaMKII expression. Sustained release of glutamate and the resulting activation of glutamate receptors increases intracellular Ca^{2+} and thereby the activity of CaMKII (Salińska et al., 2005; Vergun et al., 1999). CaMKII regulates numerous intracellular processes and is critical for normal physiological functioning of neurons (Lisman et al., 2002; Puram et al., 2011; Yamauchi, 2005). Therefore, it is important to know if R1-Pep affects the expression level of CaMKII. Untreated and glutamate stressed neurons were incubated with R1-Pep or Rand-Pep overnight and were subsequently stained for CaMKII expression. Glutamate-treated neurons displayed a significantly increased level of CaMKII (233±61% of control, Fig 4A) when compared to untreated control neurons. However, when treated with R1-Pep CaMKII levels remained unaltered under basal conditions (98±13% of control; Fig 4A) and only moderately increased after glutamate treatment (149±56% of control; Fig 4A). Although this increase in CaMKII expression was higher than in untreated control neurons, it was significantly lower than in glutamate-treated neurons with no R1-Pep-treatment (233±61% of control; Fig. 4A) or in neurons treated with Rand-Pep (228±59% of control; Fig. 4A). Together, these results indicate that treatment of neurons with R1-Pep did not alter the basal expression of CaMKII and therefore does not interfere with normal physiological activity of CaMKII.

Though R1-Pep reversed the glutamate-induced downregulation of $GABA_B$ receptors, it is essential to know if it indeed completely prevented CaMKII-mediated downregulation of the receptors via preventing the CaMKII-$GABA_B$ receptor interaction or if also other, so far unknown, CaMKII-mediated effects contribute. To test for this, we analysed the effect of R1-Pep along with the CaMKII inhibitor KN93. An additive effect of R1-Pep and K93 would indicate either incomplete inhibition of the CaMKII-$GABA_B$ receptor interaction or the involvement of additional CaMKII-mediated effects. Neurons were incubated overnight with R1-Pep or Rand-Pep and the following day the neurons were treated with KN93 and stained for cell surface expression of $GABA_B$ receptors. As observed previously (see Chapter 1), blocking CaMKII in control neurons with KN93 increased the cell surface expression of the receptors (236±64% of control; Fig. 4B). Likewise, R1-Pep-treated neurons displayed increased cell surface expression of the receptors (343±182% of control; Fig. 4B), which remained unaffected with by KN93 (320±228% of control; Fig 4B). As expected, treatment of neurons with the inactive control peptide (Rand-Pep) was without effect (basal condition: 92±30%; KN93: 239±89% of control; Fig. 4B). These results indicate that R1-Pep completely

inhibits CaMKII-mediated downregulation of $GABA_B$ receptors and ruled out any additional CaMKII-mediated effect.

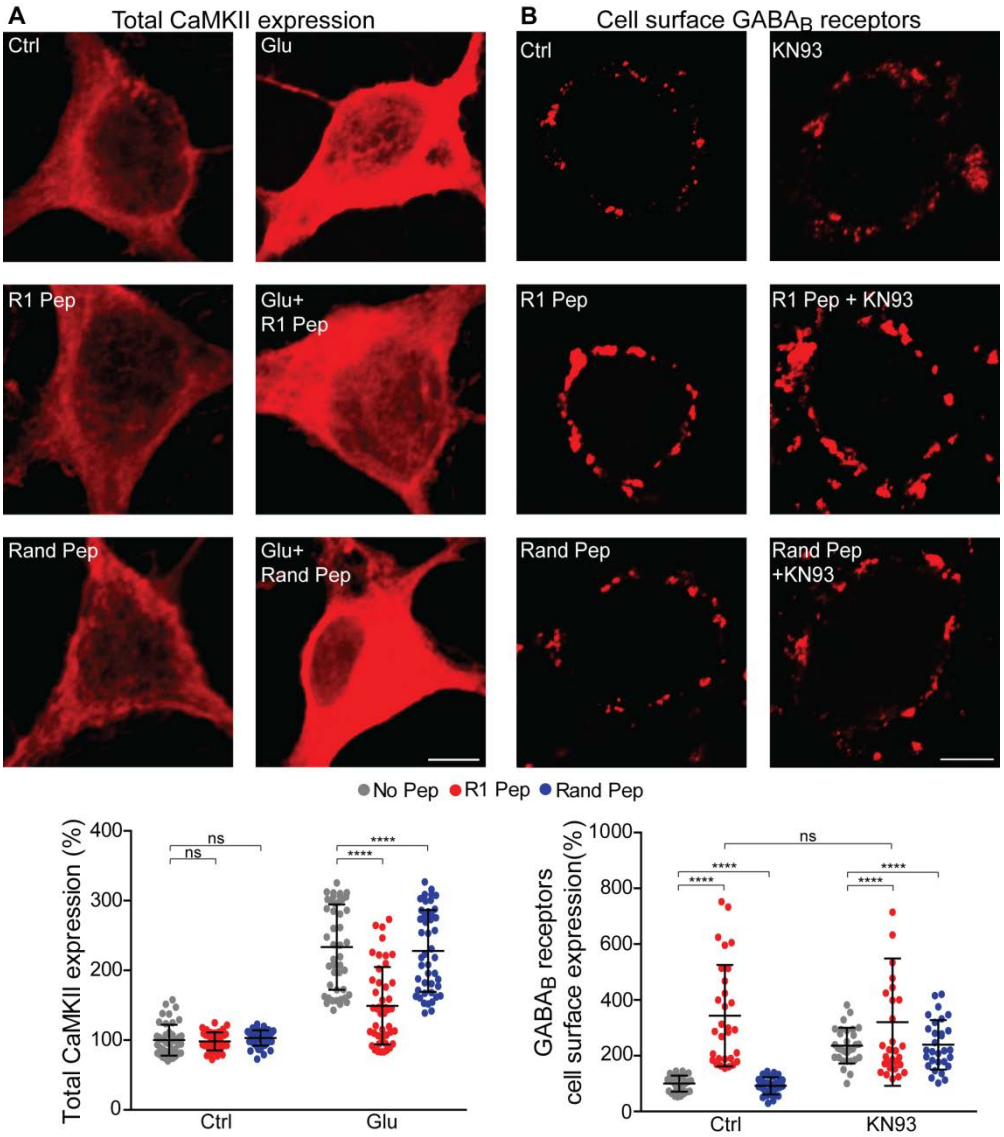


Fig: 4. *R1-Pep did not affect basal CaMKII expression (A) and the R1-Pep mediated increase in cell surface $GABA_BR$ expression is not affected further by blocking CaMKII (B).* **A.** Cultured cortical neurons were treated with glutamate (50 µM) for 60 min and immediately thereafter incubated with R1-Pep or R1-Rand overnight. The neurons were then stained for CaMKII expression. Top, representative images (scale bar, 5 µm). Bottom, quantification of fluorescence intensities. The data represent the mean ± S.D of 45 neurons per condition from two independent experiments. Two-way ANOVA with Bonferroni's Multiple Comparison test revealed an interaction between peptide treatments and glutamate stress with $F(2,264) = 24.92$, $p<0.0001$. Further, the analysis showed a significant difference in total CaMKII expression due to glutamate stress ($F(1,264) = 388.79$, $p<0.0001$) and with different peptide treatments ($F(2,264) = 29.20$, $p<0.0001$). ****, $p<0.0001$. **B.** Inhibition of CaMKII activity in addition to R1-Pep did not further increase cell surface expression of $GABA_B$ receptors. Neurons were incubated overnight (16 h) with R1-Pep or Rand-Pep and subsequently treated with KN93 (CaMKII inhibitor) for 7.5 min. Neurons were then stained for cell surface expression of $GABA_B$ receptors using $GABA_{B1b}$-N antibodies. Addition of KN93 increased cell surface expression of $GABA_B$ receptors only in neurons with no R1-Pep or with Rand-Pep and did not change the cell surface receptor in neurons with R1-Pep. Top, representative images (scale bar, 5 µm). Bottom, quantification of fluorescence intensities. The data represent the mean ± SD of 30 neurons per condition from three independent experiments. Statistical analysis with two-way ANOVA with Bonferroni's Multiple Comparison test showed an interaction between peptide treatments and KN93 treatment with $F(2,174) = 8.24$, $p=0.0004$. The analysis also showed a significant difference in cell surface $GABA_B$ receptor expression due to CaMKII inhibition by KN93 ($F(1,174) = 20.52$, $p<0.0001$) and with peptide treatment ($F(2,174) = 33.09$, $p<0.0001$). ****, $p<0.0001$.

R1-Pep prevented Lys^{63}-linked ubiquitination of GABA$_B$ receptors. CaMKII-mediated phosphorylation of $GABA_{B1}$ directs internalized receptors towards lysosomal degradation (see Chapter 1) which depends on Lys^{63}-linked ubiquitination of $GABA_{B1}$ at multiple sites (Zemoura et al., 2016). Therefore, R1-Pep should prevent Lys^{63}-linked ubiquitination of $GABA_B$ receptors. To test for this, neurons were treated with glutamate, incubated overnight with R1-Pep or Rand-Pep and then analysed for Lys^{63}-linked ubiquitination of $GABA_B$ receptors by *in situ* PLA using antibodies directed against $GABA_{B1}$ and Lys^{63}-linked ubiquitin. In agreement with our previous results (Zemoura et al., 2016), glutamate-treatment significantly increased Lys^{63}-linked ubiquitination of $GABA_B$ receptors in the absence of R1-Pep (148±63% of control; Fig. 5). As expected, R1-Pep almost completely prevented Lys^{63}-linked ubiquitination of $GABA_B$ receptors in glutamate-treated (14±16% of control; Fig. 5) and untreated control neurons (9±13% of control; Fig. 5). The inactive control peptide (Rand-Pep) did not affect Lys^{63}-linked ubiquitination of $GABA_B$ receptors (basal: 86±36% of control, glutamate: 166±84% of control; Fig. 5). These results indicate that blocking the interaction between $GABA_{B1}$ and CaMKII prevents Lys^{63}-linked ubiquitin and thereby lysosomal degradation of the receptors.

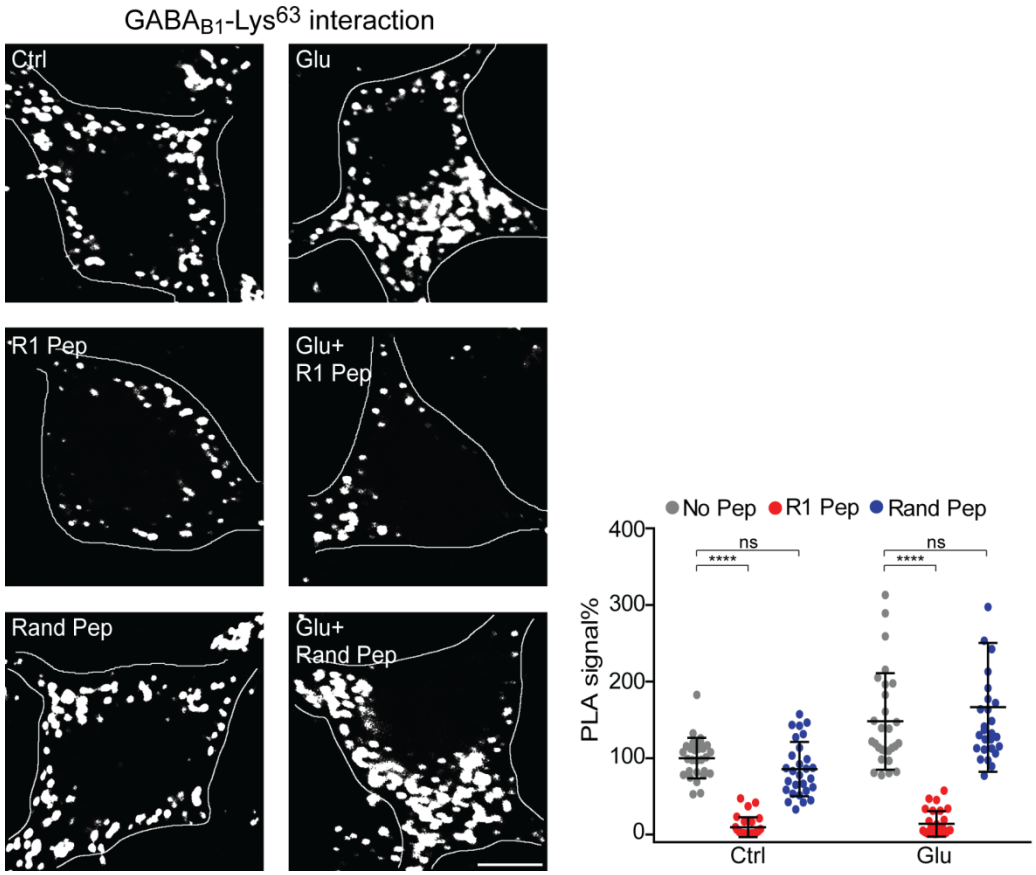


Fig: 5 *R1-Pep blocked Lys^{63}-linked ubiquitination of GABA$_B$ receptors.* The effect of R1-Pep on Lys^{63}-linked ubiquitination of GABA$_B$ receptors was analysed by *in situ* PLA using antibodies directed against Lys^{63}-linked ubiquitin and GABA$_{B1}$. Cortical neurons were stressed for 60 min with 50 µM glutamate and subsequently incubated without peptide, R1-Pep or Rand-Pep for 16 h (overnight) and subjected to *in situ* PLA. Top: representative images, *white dots* are PLA signals (scale bar, 5 µm). Treatment of neurons with R1-Pep significantly reduced Lys^{63}-linked ubiquitination of GABA$_B$ receptors. Bottom, quantification of *in situ* PLA signals. The data represent the mean ± SD of 28 neurons per condition from two independent experiments. Two-way ANOVA with Bonferroni's multiple comparison test showed an interaction between peptides treatment and glutamate stress with $F(2,162) = 9.21$, $p=0.0002$. The analysis also showed a significant difference in GABA$_{B1}$-Lys^{63} linked ubiquitin interaction due to glutamate stress ($F(1,162) = 36.75$, $p<0.0001$) and with peptides treatment ($F(2,162) = 106.18$, $p<0.0001$). ****, $p<0.0001$.

Glutamate stress downregulated the expression of GIRK channels. Important down-stream effectors of GABA$_B$ receptors are G-protein coupled inwardly rectifying K^+ channels (GIRK) (Fernández-Alacid et al., 2009; Ladera et al., 2008; Lüscher et al., 1997). As sustained glutamate receptor activation downregulates GABA$_B$ receptors, it was also likely that GIRK channels are co-regulated. To investigate this, total GIRK channel ($K_{ir}3.2$) expression was analysed in neurons treated or not with glutamate in the presence or absence of R1-Pep or Rand-Pep. Glutamate-treated neurons exhibited a significantly reduced expression of GIRK channels (58±9% of control; Fig.6), being in line with a co-regulation of GIRK channels and GABA$_B$ receptors. Treatment of unstressed neurons with R1-Pep (no glutamate-treatment) did not alter GIRK expression (107±18% of control; Fig 6). However,

R1-Pep considerably enhanced GIRK expression after glutamate stress (134±28% of control; Fig. 6). The inactive control peptide (R1-Rand) did not affect GIRK channel expression under the conditions tested (basal: 88±15%, glutamate: 52±13% of control; Fig. 6). These results indicate that R1-Pep specifically elevates GIRK channel expression only under ischemia-like excitotoxic conditions.

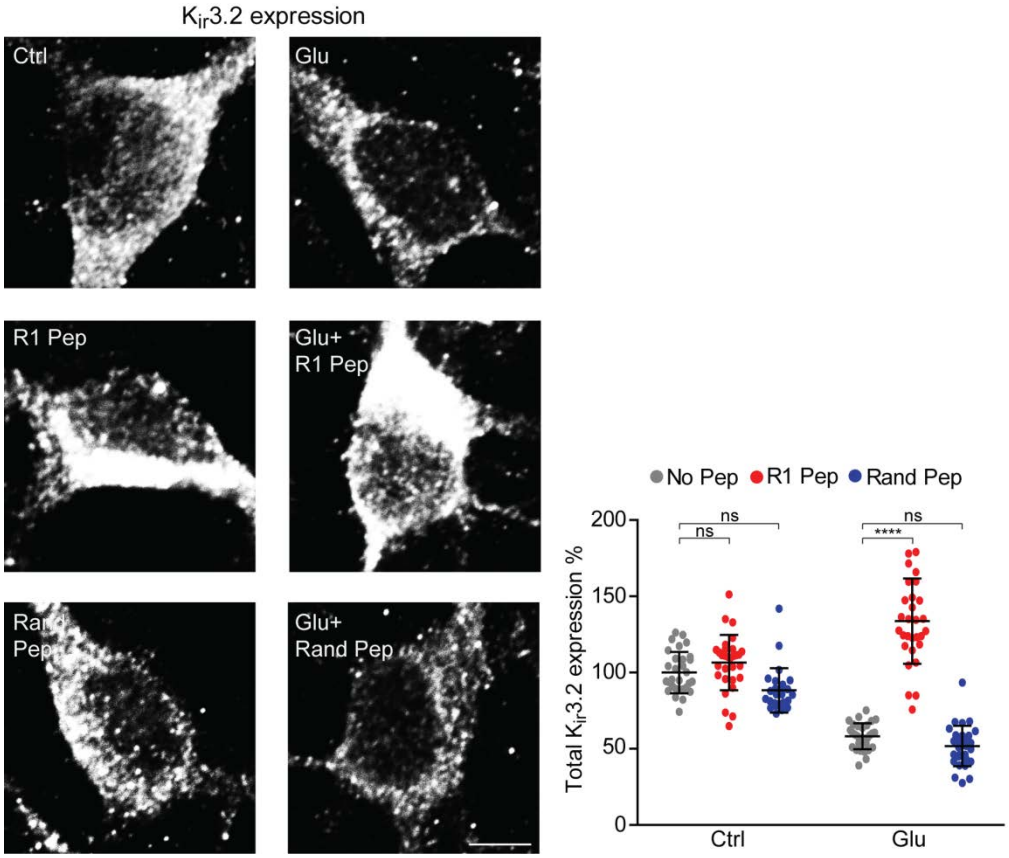


Fig: 6. *GIRK ($K_{ir}3.2$) channel expression in neurons was downregulated upon glutamate stress and increased after treatment with R1-Pep.* Cultured cortical neurons were stressed with 50 µM glutamate for 60 min and incubated immediately after the insult with no peptide, R1-Pep or Rand-Pep for 16 h (overnight). Following peptide incubation, the cells were analysed for total GIRK expression using an antibody against GIRK ($K_{ir}3.2$). Glutamate treatment reduced total GIRK expression in conditions with no peptide and Rand-Pep, while incubation with R1-pep increased total expression of GIRK channels. Top, representative images (scale bar, 5 µm). Bottom, quantification of fluorescence intensities. The data represent the mean ± S.D of 30 neurons per condition from two independent experiments. Two-way ANOVA with Bonferroni's multiple comparison test showed an interaction between peptides treatment and glutamate stress with $F(2,170) = 74.02$, $p<0.0001$. The analysis also showed a significant difference in total GIRK channel expression due to glutamate stress ($F(1,170) = 43.11$, $p<0.0001$) and with peptides treatment ($F(2,170) = 141.43$, $p<0.0001$). ****, $p<0.0001$.

Restoring cell surface expression of $GABA_B$ receptors via R1-Pep treatment reduced upregulated P/Q-type and N-type VGCC expression under excitotoxic conditions. Beside GIRK channels, voltage-gated Ca^{2+} channels (VGCC) are important down-stream effectors. $GABA_B$ receptors regulate neurotransmitter release by inhibiting P/Q-type and N-type voltage-gated Ca^{2+} channels (N. Bussières and El Manira, 1999; Cao and Tsien, 2010). As observed for GIRK channels, it is reasonable to assume that also P/Q- and N-type Ca^{2+} channels are co-regulated with $GABA_B$ receptors under ischemic-like conditions. To test this hypothesis, we treated cultured cortical neurons with glutamate (50 μM) followed by incubation with R1-Pep or Rand-Pep and analysis for total VGCC expression. Unexpectedly, we observed a strong increase in P/Q-type and N-type VGCCs expression after glutamate treatment that was not affected by the control peptide R1-Rand but was strongly decreased by R1-Pep (Table. 2, Fig. 7A, B). Interestingly, R1-Pep also considerably reduced expression of P/Q-type and N-type VGCCs in control neurons not subjected to glutamate stress (Table 2, Fig. 7A, B).

Table: 2. R1-Pep treatment reduced the expression of P/Q-type and N-type VGCCs under basal and ischemia-like conditions in cultured cortical neurons.

Type of VGCCs	No Peptide		R1-Pep		Rand-Pep	
	Control	Glutamate	Unstressed	Glutamate	Unstressed	Glutamate
P/Q-type	100±8%	214±55%	57±17%	60±17%	102±17%	207±59%
N-type	100±28%	197±34%	60±20%	65±27%	92±16%	164±41%
L-type	100±10%	158±24%	99±14%	157±22%	96±12%	154±21%

Prolonged activation of AMPA and NMDA receptors leads to an increase in the intracellular Ca^{2+} concentration by activating L-type VGCCs in the postsynaptic terminals, which induces excitotoxicity (Arundine and Tymianski, 2003; Chalifoux and Carter, 2010; Ouardouz et al., 2003; Svoboda and Sabatini, 2000). Though $GABA_B$ receptors do not directly modulate L-type Ca^{2+} channels, it was important to test if their expression is affected under glutamate stress and if this is affected by R1-Pep. In line with our finding on P/Q- and N-type Ca^{2+} channels, also the expression levels of L-type Ca^{2+} channels were upregulated after glutamate stress (Table. 3, Fig. 7C). However, neither R1-Pep nor R1-Rand affected the expression of L-type Ca^{2+} channels under basal nor glutamate conditions (Table. 3, Fig. 7C).

These results indicate that VGCCs are upregulated by glutamate stress, presumably as a homeostatic response to the insult. In contrast to GIRK channels, VGCCs are not co-regulated with $GABA_B$ receptors after glutamate stress and only the expression of the $GABA_B$ receptor regulated P/Q- and N-type Ca^{2+} channels are affected by R1-Pep.

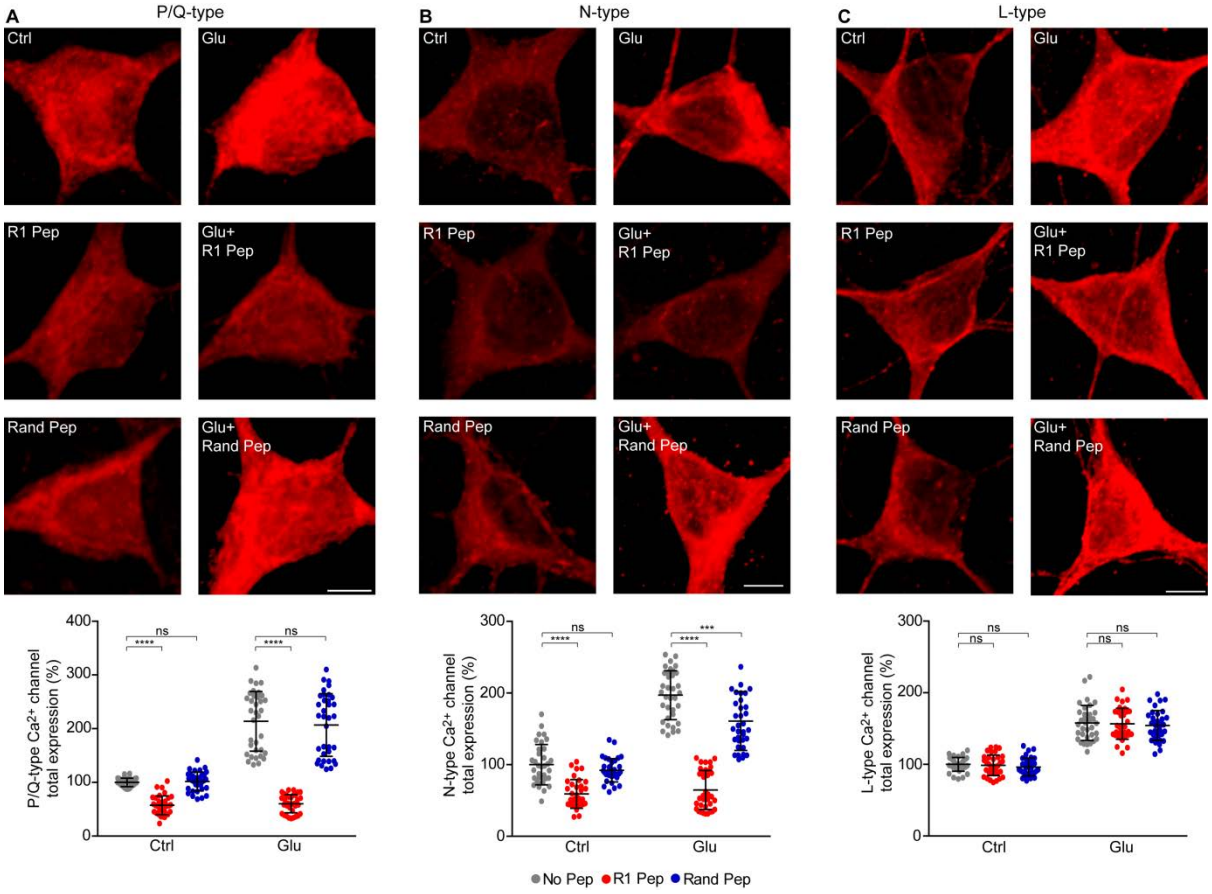


Fig: 7 *R1-Pep affected the expression of P/Q-type and N-type VGCCs but did not affect L-type VGCCs.* Cultured cortical neurons were subjected to glutamate stress (50 µM for 60 min) and immediately after the stress the neurons were treated with either R1-Pep or Rand-Pep for overnight. 16 h after addition of peptides, neurons were stained with antibodies raised against P/Q-type, N-type and L-type VGCCs to analyse for changes of their expression levels. Glutamate treatment increased the expression of P/Q-type (A), N-type (B) and L-type (C) VGCCs. R1-Pep treatment significantly reduced the expression of P/Q-type (A) and N-type (B) VGCCs, while it had no effect on the expression of L-type VGCCs (C). Top, representative images (scale bar, 5 µm). Bottom, quantification of fluorescence intensities. The data represent the mean ± SD of 35 neurons per condition from two independent experiments. Two-way ANOVA with Bonferroni's multiple comparison test showed an interaction between peptides treatment and glutamate stress for the various VGCCs evaluated (*P/Q-type* – $F(2,204) = 53.53$, *N-type* – $F(2,202) = 47.45$, $p<0.0001$) and no interaction for *L-type* – $F(2,204) = 0.00$, $p=1.000$. The analysis also showed a significant difference in VGCCs expression due to glutamate stress (*P/Q-type* – $F(1,204) = 230.72$, $p<0.0001$; *N-type* – $F(1,202) = 213.11$, $p<0.0001$ and *L-type* – $F(1,204) = 541.89$, $p<0.0001$) and with peptides treatment (*P/Q-type* – $F(2,204) = 176.19$, $p<0.0001$ and *N-type* – $F(2,202) = 173.79$, $p<0.0001$). However, the peptides treatment did not have an effect on *L-type* VGCCs expression as $F(2,204) = 0.77$ and $p=0.4642$. ****, $p<0.0001$.

R1-Pep mediated restoration of cell surface $GABA_B$ receptors activated Akt and GSK3β mediated survival cascades. Over-stimulation of NMDA and AMPA receptors activates the apoptotic cascade, leading eventually to neuronal death. In this pathway, phosphorylation of glycogen synthase kinase 3β (GSK3β) at Tyr-217 triggers activation of caspase 3 and initiates apoptosis (Endo et al., 2006; Kennedy et al., 1997; Zhao et al., 2016). On the other hand, cell survival is promoted by the phosphatidylinositol-4,5-bisphosphate 3 kinase (PI3K) / Akt (protein kinase B) pathway. PI3K activates the serine/threonine kinase Akt by phosphorylating it at S473, which in turn inhibits GSK3β by phosphorylating it at Ser-9. This inhibits the apoptogenic cascade (Endo et al., 2006; Keegan et al., 2015; Liu et al., 2015; Xu et al., 2008). It has been reported that chronic activation of $GABA_B$ receptors by baclofen induces neuroprotection in ischemic conditions via activation of the PI3K / Akt pathway, which inactivates GSK3β (Keegan et al., 2015; Liu et al., 2015)**.**

We therefore tested if the Akt/GSK3β survival cascade is activated by $GABA_B$ receptor restoration via R1-Pep after glutamate stress. For this, we determined the ratio of phosphorylated Akt (pAkt) to total Akt and the ratio of phospho-GSK3β-S9 to total GSK3β. Neurons stressed with glutamate for 60 min were incubated overnight with either no peptide, R1-Pep or Rand-Pep and were subsequently analysed for pAkt and phospho-GSK3β-S9. The ratiometric immunofluorescence analysis of pAkt/Akt indicated a significant reduction in pAkt/Akt in glutamate-treated neurons without R1-Pep (64±9% of control; Fig. 8A) and with the inactive control peptide Rand-Pep (63±11% of control; Fig. 8A). Treatment of cultured cortical neurons with R1-Pep increased pAkt/Akt irrespective of glutamate stress or not (basal: 143±22%, glutamate: 137±20% of control; Fig. 8A). Correspondingly, the ratio of pGSK3β-S9/GSK3β was significantly reduced after glutamate stress (70±15% of control; Fig. 8B), which was not affected by Rand-Pep (69±12% of control; Fig. 8B). This indicates the initiation of the apoptotic machinery after glutamate treatment. However, application of R1-Pep after glutamate stress increased the pGSK3β-S9/GSK3β ratio (138±24% of control; Fig. 8B), indicating the activation of the Akt/GSK3β-pS9 mediated survival cascade.

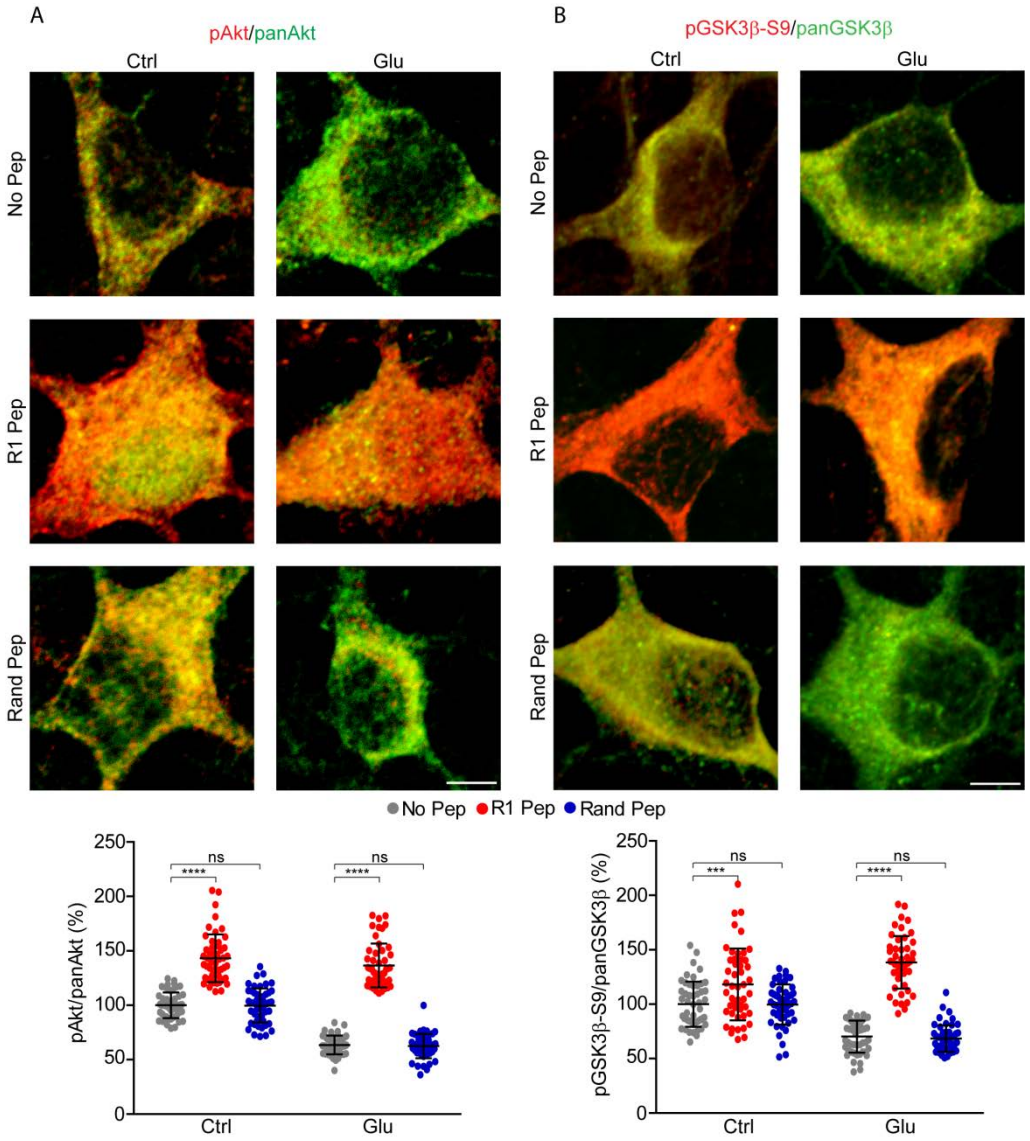


Fig. 8. *R1-Pep activated the Akt/GSK3β-pS9 survival cascade* **A.** R1-Pep increased pAkt. Cortical neurons were treated for 60 min with 50 µM glutamate and were immediately incubated thereafter with no peptide, R1-Pep or Rand-Pep for 16 h. Subsequently, the neurons were immunostained with antibodies raised against phosphorylated Akt and panAkt (total). Neurons showed an increased ratio of pAkt/panAkt after treatment with R1-Pep. Top, representative images (scale bar, 5 µm). Bottom, ratiometric quantification of fluorescence intensities. The data represent the mean ± S.D of 50 neurons per condition from two independent experiments. ***, $p \leq 0.001$; one-way ANOVA, Dunnett's Multiple Comparison test. Statistical analysis with two-way ANOVA with Bonferroni's multiple comparison test revealed a significant interaction between peptides treatment and glutamate stress with $F(2,294) = 30.74$, $p<0.0001$. Analysis also showed a significant difference in the ratio of pAkt/panAkt with glutamate stress ($F(1,294) = 217.75$, $p<0.0001$) and following peptides treatment ($F(2,294) = 460.87$, $p<0.0001$). ****, $p<0.0001$. **B.** R1-Pep increased pGSK3β-S9. Neurons were subjected to glutamate (50 µM) stress for 60 min and subsequently treated with no peptide, R1-Pep or Rand-Pep, followed by determination of pGSK3β-S9 and panGSK3β levels. R1-Pep treatment after glutamate stress increased the ratio of pGSK3β-S9/panGSK3β, indicating the activation of the survival cascade. Top, representative images (scale bar, 5 µm). Bottom, ratiometric quantification of fluorescence intensities. The data represent the mean ± S.D of ~50 neurons per condition from two independent experiments. Two-way ANOVA with Bonferroni's multiple comparison test showed an interaction between peptides treatment and glutamate stress with $F(2,292) = 45.43$, $p<0.0001$. Analysis also showed a significant difference in the ratio of pGSK3β-S9/panGSK3β, with glutamate stress ($F(1,292) = 29.56$, $p<0.0001$) and following peptides treatment ($F(2,292) = 134.51$, $p<0.0001$). ****, $p<0.0001$ and ***, $p<0.001$.

Treatment of neurons with R1-Pep limits glutamate-induced neuronal death. The previous experiment indicated that enhancement of $GABA_B$ receptor expression by R1-Pep activates the pAkt/pGSK-S9 survival cascade. Therefore, we finally tested if application of R1-Pep limits neuronal death after glutamate stress. To test this, cultured cortical neurons were subjected to glutamate stress and R1-Pep or the inactive control peptide R1-Rand was added 3 h, 6 h or 24 h thereafter. After additional 24 h, the cultures were analysed for cell death using Propidium Iodide (PI) staining (endpoint of experiment: 27 h, 30 h and 48 h, respectively). Treatment of cultured neurons with glutamate led to progressive neuronal death in the absence of peptide (32±7% dead neurons after 27 h endpoint, 53±9% after 30 h endpoint and 79±4% after 48 h endpoint; Fig. 9). A corresponding pattern of increasing neuronal death was observed in cultures treated with the inactive control peptide Rand-Pep (31±7% dead neurons after 27 h endpoint, 50±7% after 30 h endpoint and 75±5% after 48 h endpoint; Fig. 9). However, in line with the observations from the previous experiment, R1-Pep treatment following glutamate stress significantly reduced neuronal death even when added 24 h after the excitotoxic insult (16±6% dead neurons after 27 h endpoint, 22±9% after 30 h endpoint and 26±10% after 48 h endpoint; Fig. 9).

These findings verified our hypothesis that preventing downregulation of $GABA_B$ receptors under ischemic conditions limits neuronal death and revealed a very promising wide time window for the neuroprotective effect of R1-Pep.

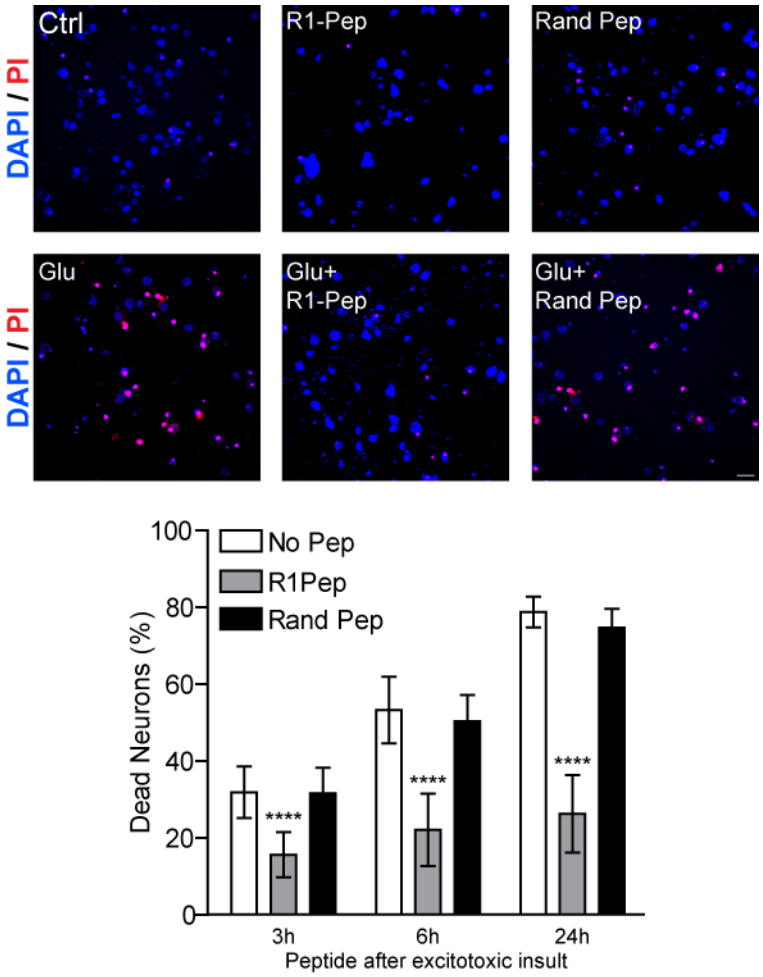


Fig. 9. *R1-Pep-induced increase in cell surface $GABA_B$ receptors limited glutamate-induced neuronal death.* Neurons were stressed with 50 μM glutamate for 60 min and treated with no peptide, R1-Pep or R1-Rand 3 h, 6 h and 24 h thereafter. After additional 24 h (i.e. after 27 h, 30 h and 48 h, respectively), the cultures were stained with Propidium Iodide (PI) for dead neurons and with DAPI for total number of neurons. The ratio of PI/DAPI positive cells was measured to calculate the percentage of dead neurons per field of view (frame). Top, representative images (6 h time point) (scale bar, 25 μm). The data represent the mean ± S.D of 15 frames per experimental condition from two independent experiments Two-way ANOVA, Bonferroni's Multiple Comparison test showed an interaction between glutamate stress and peptide treatments across various time points with $F(6,126) = 52.53$, $p<0.0001$. Also, analysis revealed a significant difference in neuronal survival with glutamate stress ($F(3,126) = 675.00$, $p<0.0001$) and with peptides treatment following stress ($F(2,42) = 296.38$, $p<0.0001$. ****, $p<0.0001$

Discussion

A strong increase in intracellular Ca^{2+} by sustained activation of glutamate receptors is the major culprit in excitotoxicity-induced neuronal death in cerebral ischemia (Forder and Tymianski, 2009; Reynolds et al., 1998). Under physiological conditions, excessive glutamate release is controlled by $GABA_B$ receptors, which are abundantly localized at the glutamatergic synapses. Hence, insufficient inhibition to control excitotoxicity could be explained by either inadequate inhibition provided by $GABA_B$ receptors at glutamatergic synapses or loss of function/expression of these receptors in affected neurons. The observation that chronic activation of $GABA_B$ receptors is neuroprotective in animal models of cerebral ischemia (Babcock et al., 2002; Fu et al., 2016; Han et al., 2008; Hao et al., 1992; Hodor et al., 2014; Hulme et al., 1985; Lal et al., 1995; Liu et al., 2015; Zhang et al., 2014, 2007), points to impaired $GABA_B$ receptor-mediated inhibition. There is in fact accumulating evidence for reduced receptor expression under ischemic conditions, which consequently would diminished $GABA_B$ receptor-mediated inhibition (Anju et al., 2011, 2010; Cimarosti et al., 2009; Kobuchi et al., 2015; Vollenweider et al., 2006). Because the maximum possible signalling strength of $GABA_B$ receptors is determined by the number of receptors present in the plasma membrane, mechanisms regulating cell surface expression are most likely affected.

Under basal conditions, $GABA_B$ receptors undergo constitutive internalization to early endosomes from which the majority of receptors traffic back to the plasma membrane and a minor fraction is sorted to lysosomes for degradation (Grampp et al., 2008, 2007; Vargas et al., 2008). However, recent *in vitro* studies suggest that sustained activation of glutamate receptors - a hallmark in cerebral ischemia - rapidly downregulates $GABA_B$ receptors by shifting the recycling/degradation balance towards degradation (Guetg et al., 2010; Kantamneni et al., 2014b; Maier et al., 2010; Terunuma et al., 2010a,b). A key step in this mechanism is the enhanced CaMKII-mediated phosphorylation of $GABA_{B1}$ at serine 867 (Guetg et al. 2010). Therefore, we hypothesized that preventing CaMKII-mediated phosphorylation of $GABA_{B1}$ might restore cell surface expression of $GABA_B$ receptors and might limit glutamate-induced excitotoxic neuronal death. Since systemically blocking glutamate receptors or CaMKII would interfere with numerous processes that regulate cellular homeostasis, we opted for an approach that specifically interferes with the interaction of CaMKII with $GABA_B$ receptors to prevent downregulation of the receptors under ischemic conditions. We expect that this strategy would predominantly affect neurons in the diseased area of the brain. To specifically prevent the CaMKII / $GABA_B$ receptor interaction we

screened for a small interfering peptide and tested its ability to prevent downregulation of the receptors and thereby to limit neuronal death under excitotoxic conditions.

We identified a peptide sequence of 22 amino acids present in the C-terminal domain of $GABA_{B1}$ that efficiently blocked the CaMKII / $GABA_{B1}$ interaction and upregulated the cell surface expression of $GABA_B$ receptors. Several commonly used tagging methods to render this peptide (R1-Pep) cell permeable, such as the TAT (Brooks et al., 2005; Futaki, 2005; Perkins et al., 1989; Torchilin, 2008; Wadia and Dowdy, 2005) or the poly-arginine sequence (Takeuchi et al., 2006) proved inefficient. Therefore, we tested a relatively new peptide sequence derived from the Rabies virus glycoprotein (RGV). This peptide sequence has been shown to deliver systemically injected cargos into brain neurons via receptor-mediated uptake (Kumar et al., 2007; Langevin, 2002). Our findings confirm those observations and show that the RVG sequence used is taken up by all cells expressing $GABA_B$ receptors at the cell surface. Therefore, this delivery mechanism is expected to ensure the delivery of the peptide to most neurons in the brain *in vivo* and moreover supports the specificity of our approach by targeting primarily $GABA_B$ receptor expressing neurons were the peptide should be active.

Our findings indicate that the R1-Pep drastically reduced the interaction between $GABA_{B1}$ and CaMKII under basal physiological conditions as well as under ischemic conditions. This was accompanied by a strong reduction of $GABA_{B1}$(S867)-phosphorylation-mediated Lys^{63}-linked ubiquitination of $GABA_{B1}$, which sorts the receptors to lysosomal degradation (Guetg et al., 2010; Maier et al., 2010; Zemoura et al., 2016), and a strong increase of cell surface expression of the receptors. These results show that preventing the interaction of $GABA_B$ receptors with CaMKII blocks the entire downstream cascade of targeting $GABA_B$ receptors to lysosomes for degradation and underscores the central role of CaMKII-mediated phosphorylation of $GABA_{B1}$ (S867) as initial signal triggering degradation of the receptors (see Chapter 1). These results also indicate that CaMKII not just regulates $GABA_B$ receptor downregulation under excitotoxic conditions but also plays a vital role in controlling $GABA_B$ receptor expression under normal physiological conditions.

We further found that the R1-Pep at low doses prevented downregulation of the receptors and at higher doses strongly increased the cell surface expression of the receptors. This observation opens the opportunity to dose-dependently adjust the cell surface expression of the receptors to achieve an optimal balance between beneficial (neuroprotection) and potential unwanted side effects (e. g. sedation).

Normal expression levels and activity of CaMKII is fundamental to physiological functioning of neurons. CaMKII regulates a variety of intracellular processes important for neuronal homeostasis (reviewed in Hell, 2014) and plays a key role in controlling long term potentiation (LTP) and long term depression (LTD), thus controlling synaptic plasticity (Coultrap et al., 2014; Coultrap and Bayer, 2012). Therefore, it is important that administration of R1-Pep does not affect CaMKII expression to avoid toxic side effects. Fortunately, our experiments ruled out any effect of R1-Pep on CaMKII expression under normal physiological conditions and hence R1-Pep should not affect other than $GABA_B$ receptor-mediated CaMKII functions. However, glutamate treatment increases CaMKII expression and translocation within the neurons that causes excitotoxicity-induced neuronal death by triggering the apoptotic cascade (Ashpole et al., 2012; Coultrap et al., 2011; Merrill et al., 2005; Sepúlveda et al., 2013). Our findings show that administration of R1-Pep after glutamate stress significantly reduced the expression of CaMKII in neuronal cultures, although not to physiological levels. This indicates that the R1-Pep mediated-increase in cell surface $GABA_B$ receptors and thereby increased $GABA_B$ receptor-mediated inhibition, also affects the mechanism responsible for upregulating CaMKII. This might also contribute to the neuroprotective effect of R1-Pep by reducing the detrimental effects overexpressed CaMKII in neurons.

One of the major downstream effector systems of postsynaptic $GABA_B$ receptors are GIRK2 (Kir 3.2) channels (Ladera et al., 2008; Lüscher et al., 1997), via which $GABA_B$ receptors control excitatory neurotransmission by hyperpolarizing the postsynaptic membrane (Padgett and Slesinger, 2010; Turecek et al., 2014). Our results show that GIRK channel expression is co-regulated with $GABA_B$ receptor expression in neurons subjected to glutamatergic stress. On sustained activation of glutamate receptors, we observed a similar decline in the expression of GIRK channels and $GABA_B$ receptors. Interestingly, in contrast to $GABA_B$ receptor expression, R1-Pep did not affect GIRK channel expression under normal physiological conditions but only upregulated GIRK channels, in neurons subjected to glutamate stress. This finding implies that signal transduction via GIRK channels, induced by other G protein coupled receptors than $GABA_B$ receptors is not compromised in healthy neurons in the presence of R1-Pep.

The other major downstream effectors of $GABA_B$ receptors at excitatory synapses are P/Q-type and N-type VGCCs that modulate glutamate release and subsequent activation of glutamate receptors (Barral et al., 2000; N Bussières and El Manira, 1999; Cao and Tsien,

2010; Chen and van den Pol, 1998; Currie and Fox, 1997; Kavalali et al., 1997; Zamponi and Currie, 2013). In addition to P/Q and N-type channels, recent studies have shown that $GABA_B$ receptors can indirectly regulate the activity of L-type VGCCs at postsynaptic locations (Chalifoux and Carter, 2011b, 2010). Interestingly, we observed a strong increase in the expression level of all three types of VGCCs after glutamate stress, which is the opposite direction of regulation as observed for $GABA_B$ receptors and GIRK channels. This upregulation of VGCCs after glutamate stress is most likely a homeostatic response caused by the increased neuronal depolarization and appears to be uncoupled from the regulation of $GABA_B$ receptors. This is a surprising finding since P/Q-type and N-type Ca^{2+} channels are physical components of the $GABA_B$ receptor signalling complex and are often co-regulated (N Bussières and El Manira, 1999; Chen and van den Pol, 1998; Currie, 2010). However, when the neurons were treated with R1-Pep after glutamate stress we observed a strongly reduced expression of P/Q-type and N-type, but not of L-type VGCCs. The reduction in expression of P/Q- and N-type VGGCs most likely results from elevated inhibitory G-protein signalling because of the increased cell surface expression of $GABA_B$ receptors. It has been shown that increased levels of Gβγ-subunits competitively displace the Ca_Vβ subunit from the VGCC complex, exposing phosphorylation sites in the Ca_V I-II loop. This triggers the internalization of the channels and subsequent degradation (Altier et al., 2006; Cantí et al., 2001; Chalovich and Eisenberg, 2005; Dolphin, 2012, 2003; Gandini et al., 2014; Hendrich et al., 2008; Page et al., 2016; Sandoz et al., 2004). Absence of any changes in the expression of L-type VGCCs after R1-Pep treatment most likely reflects a rather indirect regulation by $GABA_B$ receptors, which does not involve Gβγ (Bray and Mynlieff, 2011). These results indicate that R1-Pep tends to normalise the expression of the major relevant $GABA_B$ receptor effectors after glutamate stress.

$GABA_B$ receptors mediate neuroprotection via the PI3K / Akt-GSK3β pathway (Keegan et al., 2015; Tu et al., 2010), which is a major neuronal survival cascade (Franke, 1997; Franke et al., 1997). PI3K activates Akt by phosphorylating it at serine 473 (Alessi et al., 1996; Manning et al., 2002). Akt exerts its pro-survival functions through the phosphorylation and inhibition of BCl-2 associated death promoter (BAD) (Datta et al., 1997) and the inhibition of glycogen synthase kinase 3β (GSK-3β) (Cross et al., 1995), which prevents activation of the caspase cascade (Cardone et al., 1998). Since the R1-Pep increased cell surface expression of $GABA_B$ receptors and obviously $GABA_B$ receptor signalling, an enhanced activity of the pAkt-mediated survival cascade was expected. Indeed, our findings

indicate a strong increase in the phosphorylation of Akt at Ser-473 and GSK-3β at Ser-9 after treatment of neurons with R1-Pep under basal as well as after glutamate stress. The activation of this survival pathway via increased expression of $GABA_B$ receptors upon P1-Pep application considerably reduced neuronal death after glutamate stress. Most importantly, we found a rather wide time window for its neuroprotective effect. Even when administered 24 hrs after glutamate stress, R1 Pep limits neuronal death by almost 50%. This observation is very promising in view of the development of a potential neuroprotective therapeutic strategy for stroke patients.

Though a variety of pharmacological approaches to counteract neuronal death had been tested in clinical trials, including blocking NMDA receptors or inhibiting CaMKII, no approach was successful so far (Ashpole and Hudmon, 2011; Coultrap et al., 2011; George and Steinberg, 2015; Vest et al., 2010). In particular, global inhibition of NMDA receptors or CaMKII is toxic to neurons by itself (Ashpole et al., 2012; Hoyte et al., 2004). Hence alternate targets and approaches are needed to limit ongoing neuronal loss in the chronic phase of ischemic stroke. Cumulatively, our findings indicate that blocking the interaction of CaMKII with $GABA_B$ receptors to prevent downregulation of the receptors under ischemic conditions might be a promising and highly selective approach to limit cell death. Because the expression of the main $GABA_B$ receptor effectors (GIRK channels and VGCCs) as well as of CaMKII remained largely unaffected by R1-Pep under normal physiological conditions, we expect a rather limited profile of side effects *in vivo*.

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